Antiatherogenic effect of *Pistacia lentiscus* via GSH restoration and downregulation of CD36 mRNA expression

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Abstract

*Pistacia lentiscus* var. Chia (Anacardiaceae) grows almost exclusively on Chios Island, Greece, and gives a resinous exudate resin used for culinary purposes by Mediterranean people. We investigated the molecular mechanisms through which total polar extract of the resin inhibits oxidized low-density lipoprotein (oxLDL) cytotoxic effect on peripheral blood mononuclear cell (PBMC). Cells exposed to oxLDL underwent apoptosis and necrosis, dependent on the duration of exposure. When culturing cells with oxLDL and the polar extract concurrently, we observed inhibition of both the phenomena. Because under oxidative stress the pro-oxidant systems outbalance the antioxidant, potentially producing oxidative damage and ultimately leading to cell death, we measured the levels of intracellular antioxidant glutathione (GSH). Additionally, we measured CD36 expression, a class B scavenger receptor, on CD14-positive cells, as CD36 has been identified as the oxLDL receptor in macrophages and may play a pivotal role in atherosclerotic foam cell formation. oxLDL decreased GSH levels and upregulated CD36 expression.

*P. lentiscus* extract restored GSH levels and downregulated CD36 expression, even at the mRNA level. In order to find out the biologically drastic constituents of the resin’s polar extract, fractions derived from RP-HPLC analysis were examined for their antioxidant effect on oxidatively stressed PBMC. The triterpenoid fraction revealed remarkable increase in intracellular GSH. We suggest GSH restoration and downregulation of CD36 mRNA expression as the pathways via which *P. lentiscus* triterpenes exert antioxidant/antiatherogenic effect. Additionally, our results provide strong evidence of the resin’s antiatherogenic effect; therefore it is credited with beneficial health aspects.

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1. Introduction

Evidence has accumulated that oxidized low-density lipoprotein (oxLDL) may play an important role in the initiation and progression of atherosclerotic lesions [1]. Under oxidative stress, free radicals attack plasma LDL that is oxidatively modified leading to the attraction of blood monocytes beneath the endothelium [2]. Monocytes differentiate into macrophages that themselves effect modifications in LDL, most importantly taking up the endothelial cell modified LDL [3]. The uptake of oxLDL occurs via scavenger receptors of the class SR-A and SR-B [4], a member of which is CD36 scavenger receptor [5] that binds to its lipid moiety


In the search for compounds therapeutic against various diseases, several natural products have been investigated during the past few years, with promising prospects. Much attention has been focused on the potential antiatherogenic effects of components of the Mediterranean diet, such as red wine and olive oil. So far, in vitro and ex vivo experiments indicate that the adequate intake of phenolic compounds present in wine [8] and olive oil [9] is associated with a diminished risk of atherosclerosis. Apart from polyphenols, olive oil triterpenes have been proven as potent inhibitors of LDL oxidation in vitro [10]. Recently, the potent protective
effect of the polar extract from **Pistacia lentiscus** resin, commonly known as Chios mastic gum, against the in vitro copper sulphate-induced LDL oxidation has been well documented [11]. Triterpenes present in **P. lentiscus** essential oil exhibit remarkable antioxidant effect on LDL [11]. **P. lentiscus** var. Chia grows almost exclusively in Chios Island (Greece) and gives resins exude resin after longitudinal incisions at close intervals from the base of the trunk up to the thicker branches of the tree. Chios mastic gum is basically consumed as chewing gum and also in other culinary art usage, especially in Greek, Turkish and Arabic kitchens, i.e. in the powder form as food additive, in the form of sugar containing gel as a sweetener and as mastic oil as a sweet additive in drinks. It has been referred to over centuries as having medicinal properties to treat a variety of diseases. It has been proven as a therapeutic agent against various gastric malfunctions, such as gastralgia, dyspepsia and gastric ulcer [12]. In 1999, Huwel et al. [13] published the potentiality of the resin to kill the bacteria *Helicobacter pylori*. In 2002, the hepatoprotective effect of the aqueous extract from the leaves of **P. lentiscus** on CCl4 intoxicated rats was published [14]. To date though, no investigations have been carried out to highlight the activity of the resin on peripheral blood mononuclear cells (PBMCs) under oxidative stress. Monocytes are strongly implicated in atherogenesis [15] and are critical for T cell survival in the presence of oxLDL [16]. Thus, the aim of this study was to investigate the effect of total polar extract from **P. lentiscus** resin on the survival of oxLDL-treated PBMC. To determine this effect, we assayed PBMC to MTT and annexin V binding assays. Because DNA laddering is characteristic of programmed cell death, cell DNA was isolated and size-fractionated by gel electrophoresis. In an attempt to elucidate the mechanism, we measured glutathione (GSH) levels as it is an intracellular detoxification agent of toxic compounds and the oxLDL receptor CD36 expression, even at the transcriptional level. Finally, for the determination of the most drastic constituents of the resin’s polar extract, fractions derived from RP-HPLC analysis were examined for their effect on oxidatively stressed PBMC.

2. Materials and methods

2.1. Materials

- Sterilized LDL (lyophilized from 1 ml of LDL solution in 0.15 M NaCl and 0.01% EDTA at pH 7.4), phosphate buffer saline (PBS) tablets, Ficoll-Hypaque, acridine orange fluorescent dye (A-6014), ethidium bromide (E-8751) and MTT were purchased from Sigma Co. (St. Louis, MO, USA). The commercial LDL used in all experiments was crude normal, collected during spring 2003 and kindly donated from Chios Gum Mastic Growers’ Association. All the resin given was extracted according to the solvent sequence methanol/water 60:40 (v/v) and methanol on the same day.
- In brief, 1.0 ml of working-LDL was mixed with 1.0 ml of CuSO4 (10 μM) as oxidizing agent and incubated at 37°C for 6 h. The solution containing the resulted oxLDL was kept at −8°C until use. To measure lipid peroxidation, three independent LDL oxidation tests were performed and afterwards the resulted suspensions were subjected to thio-barbituric acid (TBA) test. The TBA reactant substances (TBARS), mainly malonaldehyde, resulting from oxidation, were measured spectrophotometrically at 532 nm, after re-action with 2 ml of 1% alkaline TBA solution. TBARS were measured spectrophotometrically at 532 nm, after re-action with 2 ml of 1% alkaline TBA solution.

2.2. Extraction of **P. lentiscus** resin

**P. lentiscus** resin used in all experiments was crude normal, collected during spring 2003 and kindly donated from Chios Gum Mastic Growers’ Association. All the resin given was extracted according to the solvent sequence methanol/water 60:40 (v/v) and methanol on the same day.
- Pooled and MTT were purchased from Sigma Co. (St. Louis, MO, USA). The commercial LDL used in all experiments was crude normal, collected during spring 2003 and kindly donated from Chios Gum Mastic Growers’ Association. All the resin given was extracted according to the solvent sequence methanol/water 60:40 (v/v) and methanol on the same day.

2.3. LDL modification

Commercial LDL was kept at −8°C and a working suspension (200 μg protein/ml PBS) was prepared just before usage. The LDL oxidation test was performed as has been previously described [18] with slight modifications. In brief, 1.0 ml of working-LDL was mixed with 1.0 ml CuSO4 (10 μM) as oxidizing agent and incubated at 37°C for 6 h. The solution containing the resulted oxLDL was kept at −8°C until use. To measure lipid peroxidation, three independent LDL oxidation tests were performed and afterwards the resulted suspensions were subjected to thio-barbituric acid (TBA) test. The TBA reactant substances (TBARS), mainly malonaldehyde, resulting from oxidation, were measured spectrophotometrically at 532 nm, after re-action with 2 ml of 1% alkaline TBA solution. TBARS were measured spectrophotometrically at 532 nm, after re-action with 2 ml of 1% alkaline TBA solution.
evaluated using a standard curve of standard malonaldehyde in different concentrations versus absorption at 532 nm and were quantified at 2.69 (±0.34) μM malonaldehyde.

2.4. Cell culture
PBMC (50–60 × 10^6) were isolated from healthy human volunteers as has been previously described [19]. Afterwards, they were resuspended in complete medium consisting of RPMI 1640 medium supplemented with 10% FCS, 1% L-glutamine and 1% penicillin/streptomycin. PBMC were added to each well of a 24-well plate at a density of 2 × 10^6 cells/ml and at final volume 2 ml, either in medium only (control), or with oxLDL (the solution of which had just been evaporated under a stream of nitrogen and resuspended in 1 ml of the above-described complete medium), or with oxLDL (treated as above) and concentrated polar extract from P. lentiscus resin of known FC reactant substances. Individual 2 μl extract volumes containing 5.4, 54 and 540 μg of FC reactant substances (equivalent to cafefeic acid) were added in individual cell culture of 2 ml medium, resulting in the desirable concentration of FC reactant substances (that is 2.7, 27 and 270 μg/ml) were added in individual cell culture of 2 ml medium and at final volume 2 ml, either in medium only (control), or with oxLDL (the solution of which had just been evaporated under a stream of nitrogen and resuspended in 1 ml of the above-described complete medium), or with oxLDL (treated as above) and concentrated polar extract from P. lentiscus resin of known FC reactant substances. Individual 2 μl extract volumes containing 5.4, 54 and 540 μg of FC reactant substances (equivalent to cafefeic acid) were added in individual cell culture of 2 ml medium, resulting in the desirable concentration of FC reactant substances (that is 2.7, 27 and 270 μg/ml). Dilution of methanol to cell medium was chosen 1/1000 (v/v) in order to eliminate the effect of methanol. Cells were cultured in a humidified cell incubator under 5% CO2 and 37°C in a RPMI-1640 medium (Gibco, Lonza) supplemented with 10% FCS, L-glutamine and 1% penicillin/streptomycin.

2.5. Cell viability assay
Effect on cellular viability was evaluated via MTT assay as has been previously described [20]. The optical density of the cellular homogenate was measured at 550 nm using an Elisa reader (Versamax). As a background value, a well containing only complete medium plus MTT plus isopropanol was used. Each experiment was carried out in triplicate. Fractional absorbance was calculated using the equation:

Fractional absorbance = Absorbance in test wells - Absorbance in background well / Absorbance in control wells - Absorbance in background well

2.6. Annexin V binding assay
Percentages of apoptotic and necrotic cells were assayed by annexin V-FITC and propidium iodide (PI)-staining on a Becton & Dickinson FACScan. Measurements were performed using the apoptosis detection kit as recommended by the manufacturer. Each experiment was carried out in triplicate.

2.7. Fluorescence microscopy
To distinguish living from apoptotic and dead cells, PBMC, of the same cell cultures as those disposed to annexin V binding assay, were stained with acridine orange fluorescent dye (100 μg/ml) mixed with ethidium bromide (100 μg/ml) in PBS-bovine serum albumin (1%) and were immediately examined under a fluorescence microscope (Leica, DC 300F).

2.8. Analysis of DNA fragmentation
For the analysis of DNA laddering, characteristic of programmed cell death, DNA was isolated from 2 × 10^6 cells using a DNA laddering kit as indicated by the manufacturer.

2.9. Flow cytometric analysis of CD36
At the end of incubation, 10^5 cells were washed with PBS and afterwards double-dyed stained with PE-labeled CD14 and FITC-labeled CD36 for 30 min at 4°C. The cells were then washed twice with PBS to remove the bound antibodies and were immediately analyzed on a FACScan. Monocyte population was gated and percentage of CD36 on CD14-positive cells was measured using CellQuest software. Each experiment was carried out in triplicate.

2.10. Assay for GSH
At the end of incubation, cells were extracted with 5% metaphosphoric acid solution and then centrifuged at 2000 × g for 10 min. The resulting supernatant was separated and the GSH assay was performed using the colorimetric assay for glutathione as indicated by the manufacturer. GSH concentration was evaluated using a standard curve of absorbance units versus GSH concentrations and expressed as μM. Each experiment was carried out in triplicate.

2.11. Quantification of mRNA expression
Total RNA was extracted using a phenol/guanidine isothiocyanate method according to the manufacturers’ instruction. Briefly, PBMC were precipitated and lyzed in 1 ml of Trizol® reagent and stored at –70°C until used. One half of the lysate was then subjected to one round of chloroform extraction, isopropanol precipitation, 70% ethanol wash, and the RNA was then vacuum dried and resuspended in 10 μl RNase-free water. Reverse transcription was performed at 37°C in a 50 mM Tris-HCl, 75 mM KCl, 3 mM MgCl2 buffer containing 10 mM DTT, 0.4 mM dNTPs, 0.5 μg random hexamer primers and 200 units of reverse transcriptase (Superscript™, Invitrogen). cDNA was stored at −20°C until used as template for semiquantitative RT-PCR. To that end, the cDNA was PCR amplified in the presence of 2.5 mM MgCl2, 400 μM dNTPs, 2.5 units Taq polymerase and 0.25 μM of the following primer pairs:
CD36L: 5′-CAGCCCAATGGGACCAC-3′ and CD36R: 5′-CAGGCTAGATAGACCTGC-3′, amplifying a 487 bp fragment of the CD36 gene [21] and actin-sense: 5′-TGA¬
CGGGGTCATCCACACTGTGCCCATCTA-3′ and actin-
 antisense: 5′-CTAGAGCATGACGATGACAGATGGAGG-
GG-3′, amplifying a 661 bp fragment of the β-actin gene. Conditions used were 35 cycles of 94 °C for 40 s, 55 °C for 40 s and 72 °C for 40 s for CD36 and 30 cycles of 94 °C for 40 s, 50 °C for 40 s and 72 °C for 1 min for β-actin amplifica-
tion. Subsequently, 20% of the PCR reaction volume was run in a 1.8% agarose gel. Band densities were measured and analyzed with the BandLeader™ V3.00 software. The ratios of CD36 to β-actin densities are considered to reflect relative CD36 mRNA abundance. Each experiment was carried out in triplicate.

2.12. HPLC analysis

Isolation of the resin polar extract fractions was accom-
plished by RP-HPLC. An HPLC system (Agilent Technolo-
gies, model HP 1050, Waldbronn, Germany) combined with
auto-sampler, diode array detector (HP-1050), fluorescence
spectrophotometer was used (HP 1046A) and data software was used. Analysis of P. lentiscus extract and isolation of fractions was per-
formed as previously described [22] slightly modified for the present study [23]. Briefly, a quaternary solvent system
was used consisting of water, methanol, acetonitrile and iso-
propanol, with gradient elution on a Nucleosil C18 column (Macherey-Nagel, Düren, Ger-
many). Injections of 100 μl from a solution of P. lentiscus
extract in methanol were performed.

2.13. GC–MS analysis

Fractions from HPLC were evaporated under a stream of
nitrogen. Trimethylsilyl derivatives were prepared after re-
action of dry residues with BSTFA and TMCS at 70 °C for
30 min. GC–MS data were obtained with a fused silica 5%
phenyl–95% methyl siloxane column (30 m × 0.25 mm i.d. 
× 0.25 μm film thickness), in a gas chromatograph (HP 6890)
coupled to the ion source of a MSD (HP 5972). Helium was
used as carrier gas with a linear velocity of 27 cm/s. The tem-
perature was programmed at 70 °C for 5 min, then ramped
at the rate 15 °C/min to 130 °C and subsequently increased to
4 °C/min to 170 °C where it remained for 15 min. Finally,
temperature was ramped to 300 °C at a rate of 10 °C/min
and remained to this final point for 30 min. The mass spec-
trometer was scanned from 50 to 700 m/z and ions were
generated by EI (70 eV). MS information was interpreted and
compared to Nist Mass Spectral Library.

2.14. Evaluation of the biologically drastic compounds

To find out whether the effect was due to the polyfenolic
content or due to the triterpenoid content of the resin, cells
were cultured under the above-mentioned culture conditions
with oxLDL and each of the fractions derived from HPLC.
After the end of incubation, the supernatants collected were
subjected to GSH assay.

2.15. Statistical analysis

Data were expressed as means ± S.D. Comparisons were
determined using the paired t-test. Differences between the
means were considered statistically significant at P < 0.05.

3. Results

3.1. Effect of P. lentiscus resin extract on oxLDL-induced
cytotoxicity

Cytotoxicity of oxLDL on PBMC and resistance when ex-
posed to the extract were assayed via MTT assay. As shown
in Fig. 1, for 48 h in culture, oxLDL revealed cytotoxic effect
on PBMC resulting in significant decrease in their number
to 60.1 ± 2.0% living cells. Fig. 1 also leads us to conclude
that addition of 2.7 μg/ml of the extract inhibits cytotoxic-
ity to some extent (63.2 ± 3.0% survival), while 270 μg/ml
medium of the polar extract inhibits cytotoxicity extensively
(95±3.2% survival). Ten-fold decrease to 27 μg/ml medium
exhibits similar protection to revealed at the higher concen-
tration revealed (90.0 ± 2.4% survival). Therefore, concen-
tration equivalent to 27 μg/ml of the extract was used in all
subsequent experiments. Fig. 2 indicates that when expos-
ing PBMC to oxLDL for 48 and 72 h, the minimum and
maximum lengths of time selected respectively, living cells
decreased to 64.5 ± 2.7% and 66.1 ± 3.1% in that order. In
the presence of 27 μg/ml, cytotoxicity was inhibited almost
totally. Inhibitory effect of the extract on cytotoxicity was
in all cases statistically significant.

Fig. 1. Effect of different concentrations of Folin Ciocalteau (FC) reactant
substances in the polar extract from P. lentiscus resin (P.L.) on oxidized
LDL (oxLDL): cytotoxicity against PBMC. Cell viability was assessed via
the MTT assay. Values are means±S.D. of three independent experiments.
Asterisk (∗) points out statistically (P < 0.05) significant results.
Fig. 2. Time-dependent effect of 27 μg/ml Folin Ciocalteau (FC) reactant substances in the polar extract from P. lentiscus resin (P.L.) on oxidized LDL (oxLDL) cytotoxicity against PBMC. Cell viability was assessed via MTT assay. Values are means ± S.D. of three independent experiments. Asterisk (*) points out statistically (p < 0.05) significant results.

3.2. Inhibition of oxLDL mediated apoptosis and necrosis by P. lentiscus resin extract

To examine the effect of P. lentiscus extract on survival of PBMC, we assayed cultured cells for the ability to undergo apoptosis or necrosis. Annexin exhibits selective affinity for negatively charged phospholipids of the apoptotic cell membranes, while PI stains necrotic cells with disruptive internal and external membranes. Under salt and calcium concentrations, annexin V is predisposed to bind phosphatidylserine over most other phospholipids species present in many sites on the cell surface, therefore resulting in a very intense signal. In Fig. 3, flow cytometric analysis of annexin V and PI labeled PBMC indicated that cells exposed to neither oxLDL nor polar extract for 48 h were 99.6% living (Fig. 3A), whereas when exposed to oxLDL for 48 h were 9.36% apoptotic and 4.58% necrotic (Fig. 3B). On the other hand, PBMC cultured under oxidative and extract conditions for 48 h simultaneously were 0.55% positive for annexin and 2.87% positive for PI (Fig. 3C). In Fig. 4 where PBMC cultured for 72 h are presented, under no agent 99.2% were alive (Fig. 4A), while under oxLDL only 0.82% underwent apoptosis and 73.5% underwent necrosis (Fig. 4B). Inhibition of the phenomenon of necrosis to 4.04% cells was detected under oxidative and extract conditions (Fig. 4C), indicating the antioxidant effect of P. lentiscus polar extract. We also checked oxLDL and polar extract effect on PBMC by analysis of DNA fragmentation. As shown in Fig. 5, the classical DNA ladder assay shown by gel electrophoresis did not reveal any apoptotic pattern.
in oxLDL-treated PBMC. However, the absence of DNA degradation is perhaps due to a progressive loss of cleaved DNA from cells. DNA electrophoresis analysis may not determine whether cells undergo apoptosis since it does not lend itself to rapid multiparameter analysis at the single cell level. DNA laddering is a late apoptotic event. In the present study, the number of late apoptotic cells (0.98 out of 9.36%, Fig. 3) is too low to justify a DNA laddering pattern. Early apoptotic cells potentially do not present a DNA laddering pattern. Fluorescence microscopy, a non-quantitative assay of analysis, relies dependability upon dyes that stain either viable or non-viable cells. To observe microscopically the effect of \textit{P. lentiscus} polar extract on PBMC under oxidative stress, cells were stained with acridine orange and ethidium bromide. Both dyes intercalate into DNA and stain nuclei green and orange, respectively. Viable cells have intact nuclei and stain green. Apoptotic cells have very visible piknotic nuclei with DNA condensation and stain yellow. Necrotic cells fluoresce orange.

**3.4. CD36 mRNA expression**

Results of CD36 mRNA expression are given in Figs. 9 and 10. Control populations expressed CD36 at the mRNA level (Fig. 9, lanes A and B) that was upregulated in the presence of oxLDL (Fig. 9, lanes C and D). Extra bands in lanes C and D are perhaps due to non-specific PCR amplification of similar to CD36 molecules after the oxLDL induction. Incubation of cells with oxLDL and the polar extract concomitantly, released significant decrease in mRNA expression (Fig. 9, lanes E and F). That is, after 48 h in culture, oxLDL raised CD36 mRNA expression from 35 to 75%, while simultaneous presence of total polar extract from \textit{P. lentiscus} constrained CD36 mRNA expression to 8% (Fig. 10). Similarly, after 72 h in culture, presence of oxLDL increased message for CD36 from 81 to 100%, whereas polar extract inhibited CD36 expression at the mRNA level to 6% (Fig. 10).

**3.6. Biologically drastic compounds**

Based on both the HPLC and GC chromatographic profile of polyphenolic and triterpenoid standards, the first HPLC fraction obtained was considered to contain the resin polyphenols and the second the triterpenoids. For the purpose of verification and characterization of each fraction, GC-MS analysis was performed [22]. Measurement of GSH levels after PBMC treatment individually with the polyphenolic and triterpenoid HPLC fractions revealed an increase in GSH 5.0% when treating cells with oxLDL and...
Fig. 6. Fluorescence microscopy of acridine orange- and ethidium bromide-stained PBMC. PBMC were cultured for 48 h (A–C) and 72 h (D, E, F) in the absence of any agent (A, D), with oxidized LDL (oxLDL) (B, E) and with oxLDL and 27 μg/ml Folin Ciocalteau (FC) reactant substances in the polar extract from P. lentiscus resin (P.L.) (C, F).
the polyphenolic fraction and 23.6% when treating cells with oxLDL and the triterpenoid fraction, compared to GSH measured when culturing cells with oxLDL alone (Fig. 11).

4. Discussion

In the present paper, it is demonstrated for the first time that enrichment of oxLDL cultured PBMC with the polar extract from *P. lentiscus* is associated with diminished cell mortality. Evidence of the antiatherogenic effects of several antioxidants, including polyphenols or triterpenes, on crucial cells implicated in atherogenesis, such as endothelial cells, platelets, smooth muscle cells and macrophages, is nowadays mounting. Among other constantly shown antioxidant properties of polyphenols and triterpenes, most in vitro studies indicate protective effect against LDL oxidation [10,11,24]. Apart from the aforementioned in vitro antioxidant effect of the polar extract from *P. lentiscus* resin [11], it has also been shown that the polar saliva-extract of the resin obtained when chewing it as a gum exhibits protective effect against LDL oxidation as well [25]. Consistently with these findings, investigation of the effect of the resin’s polar extract on PBMC response to oxLDL is important, as it sheds light on its possible antiatherogenic properties.

Hereby, it is shown, by different assays, that oxLDL is highly cytotoxic on total PBMC population. Because we used copper sulphate (5 μM final concentration) to modify LDL to its oxidized form, PBMC were also cultured with copper sulphate in the same final concentration. Neither MTT, nor annexin V binding assay showed any effect of copper sulphate on cells (data not shown), thus eliminating any possibility that copper sulphate interfered to cytotoxicity itself. Our findings point out that oxLDL owes cytotoxicity to decrease in GSH. This is in accordance with the recently published results [26] showing that oxLDL is primarily responsible for GSH depletion creating an oxidizing environment required for γ-GSC induction and compensatory GSH synthesis. Ghiselli et al. [27] have shown that GSH decrease occurs during cell death through a physiological process, i.e.
Fig. 9. Effect of the polar extract (27 mg/ml) from *P. lentiscus* resin enrichment of cells on CD36 mRNA. CD36 mRNA was quantified by RT-PCR, using β-actin as control. PBMC cultured in the absence of oxidized LDL (oxLDL) and Folin Ciocalteau (FC) reactant substances in the polar extract from *P. lentiscus* resin (P.L.) for 48 h (lane A) and for 72 h (lane B); PBMC cultured with oxLDL for 48 h (lane C) and for 72 h (lane D); PBMC cultured with oxLDL and FC reactant substances in the polar extract from P.L. resin for 48 h (lane E) and for 72 h (lane F). Lane m: marker.

Fig. 10. Time course effect of the polar extract [27 µg/ml Folin Ciocalteau (FC) reactant substances] from *P. lentiscus* resin (P.L.) on oxidized LDL (oxLDL)-induced CD36 mRNA expression. Values are means ± S.D. of three independent experiments. Asterisk (*) points out statistically (*P < 0.05) significant results.
Effect of the triterpenoid and polyphenolic fraction of the polar extract from *P. lentiscus* resin (P.L.) on oxidized LDL (oxLDL)-induced glutathione (GSH) depletion. Values are means ± S.D. of three independent experiments. Asterisk (*) points out statistically (*P* < 0.05) significant results.

Our findings suggest that PBMC undergo severe damage when subjected to oxLDL. In accordance with previous work [28], it is observed that cellular responses to oxLDL were dependent on the duration of exposure. That is, apoptosis for 48 h exposure and severe necrosis for 72 h exposure.

As far as monocytes are concerned, loading with oxLDL significantly increased CD36 expression. Feng et al. [29] reported that monocyte CD36 expression is upregulated in the presence of oxLDL and IL-4 by a common signaling pathway dependent on protein kinase C and PPAR-γ. Devaraj et al. [21] observed that CD36 expression is upregulated indeed, however after 96 h in culture, and gets maximal (five-fold) after 8–10 days. We show that 5% increment in CD36 expression occurs after 72 h in culture, indicative of ongoing monocyte-to-macrophage differentiation. It was also examined whether the expression of CD36 was due to respective message for CD36. Compliant with that study, we show that CD36 mRNA expression was upregulated in the presence of oxLDL, meaning that expression increases even at the transcriptional level. The significance of enhanced CD36 expression is due to the fact that once macrophages attract oxLDL to scavenger receptors, they promote endocytosis and convert into cholesterol-loaded foam cells. In our study, the reduced uptake of oxLDL might be attributed to the downregulation of CD36 in the presence of the extract, both at the protein and the mRNA levels.

In conflict with the above-mentioned oxLDL effects, the polar extract from *P. lentiscus* resin was proven to inhibit cytotoxicity, both apoptosis and necrosis, restore GSH levels, downregulate CD36 expression even at the transcriptional level. To clarify whether the extract’s effect was on oxLDL or cells, PBMC were subjected to (a) oxLDL for 24 h and (b) oxLDL for 24 h, cells were washed to collect the supernatant and then cultured for another 24 h with the polar extract. At the end of incubation, GSH assay was performed in both cultures revealing 22.0% decrease under oxidative conditions compared to the control, but total restoration when removing oxLDL and adding the polar extract (data not shown). The result drove us to conclude that the extract is drastic on PBMC rather on oxLDL.

Interestingly enough, when comparing the FC reactant content in extract from *P. lentiscus* of different time collection, different values are obtained (data unpublished, manuscript in preparation). This observation led us to use the extract of a certain portion of 2003 springtime collection. The identification and quantification of polyphenols and triterpenes in the polar extract of the resin has been recently published by our team [23], while triterpenes have been broadly investigated in the past by Papageorgiou et al. [30]. Among the constituents remained in the polar extract, triterpenes were the most active on the antioxidant defence of PBMC. Mass spectra indicated oleanolic acid and urs-12-en-28-al as the major components of the triterpenoid fraction (data not shown) while indicated in the polyphenolic fraction were tyrosol, *p*-hydroxy-benzoic acid, *p*-hydroxy-phenylacetic acid, vanillic acid and traces of gallic and *trans*-cinnamic acids. Oleanolic acid and iso-mer ursolic acid have been found to increase glutathione and superoxide dismutase in Dahl salt-sensitive insulin resistant rat model of genetic hypertension [31]. They have been also proven to exhibit antioxidant effect [11], antihyperlipidemic and antihypertensive effects and to prevent the development of atherosclerosis [32]. Regarding polyphenols, these have been proven to be potent antioxidants [33].

Bearing all in mind, the resin enhances cell defense against oxidative stress via pivotal physiological pathways, i.e. GSH and CD36, rather than counteracts oxLDL. Up to now, little did we know about its biological properties. However, this study is the first to indicate a new potential therapeutic use, beyond the antiulcer one, of this species, owed mainly to its triterpenoid content. *P. lentiscus* resin is indicated as a novel antioxidant/antiatherogenic agent of the Mediterranean areas.
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References


